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Assembly of Recombinant Human Immunodeficiency Virus Type 1 Capsid Protein In Vitro

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The capsid protein (CA) (p24) of human immunodeficiency virus (HIV) type 1 expressed in *Escherichia coli* and purified to >90% homogeneity was used to examine assembly in vitro and to probe the nature of interactions involved in the formation of capsid structures. The protein was detected in dimeric and oligomeric forms as indicated by molecular size measurements by gel filtration column chromatography, sedimentation through sucrose, and nondenaturing gel electrophoresis. Chemical cross-linking of CA molecules was observed with several homobifunctional reagents. Oligomer size was dependent on cross-linker concentration and exhibited a nonrandom pattern in which dimers and tetramers were more abundant than trimers and pentamers. Oligomers as large as dodecamers were detected in native polyacrylamide gels. These were stable in solutions of high ionic strength or in the presence of nonionic detergent, indicating that strong interactions were involved in oligomer stabilization. Limited tryptic digestion converted the putative dodecamers to octamers, suggesting that a region involved in CA protein multimerization was exposed in the structure. This region was mapped to the central portion of the protein. The recombinant CA proteins assembled in vitro into long rodlike structures and were disassembled into small irregular spheres by alterations in ionic strength and pH. The observation that assembly and disassembly of purified HIV type 1 CA protein can be induced in vitro suggests an approach for identifying possible control mechanisms involved in HIV viral core assembly.

Human immunodeficiency virus (HIV), the causative agent of AIDS, is a member of the lentivirus group of the family *Retroviridae* (9). In these spherical enveloped viruses, the viral genome is located inside a core structure that is encased in an envelope consisting of host-derived membrane and integrated viral transmembrane and surface glycoproteins (15). As with other retroviruses, the formation of virus involves at least two major assembly processes, one for the viral envelope and the other for the viral core (5, 9, 51).

Synthesis, processing, and glycosylation of the envelope precursor occur in the endoplasmic reticulum. The envelope proteins are transported to the cell plasma membrane via the secretory pathway (10). Structural proteins and enzymes that are found in the core are synthesized in the cytoplasm as two polyprotein precursors: Gag and Gag-Pol. The 55-kDa Gag polyprotein encoded by HIV contains domains for matrix (MA), capsid (CA), nucleocapsid (NC), and p6, a domain unique to lentiviruses (36). Occasional ribosomal frameshifting at a site within the NC-p6 intergenic region results in the synthesis of a polyprotein (160 kDa) that contains *pol*-encoded domains for the enzymes proteinase (PR), reverse transcriptase (RT), and integrase (IN) in addition to the *gag*-encoded domains MA, CA, and NC (25, 31). Translation and cotranslational myristylation of these polyproteins are followed by an assembly stage which consists of several temporally ordered events (5, 9, 51). Both polyproteins are transported to the plasma membrane, where assembly takes place on the cytoplasmic side of the cell membrane simultaneously with the budding and release of immature viral particles. Budding or released particles undergo a maturation process that results in the formation of mature infectious virus (8, 26, 40, 46).

The assembly phase is divided into two major stages which are characterized by the morphology of the structure found inside the viral envelope. The electron-dense structure seen in immature particles is spherical and lines the inside of the viral envelope, while the structure seen in mature virus is cone-shaped and eccentrically located (6, 14, 15, 23, 24, 52). Expression of various HIV *gag* constructs has demonstrated that the Gag precursor polyprotein can, by itself, form spherical structures like those found in immature particles (16). Formation of the immature particle and its conversion to mature virus are sensitive to mutations in *gag* and *pol* genes, indicating that regions in Gag and Gag-Pol contain information required for various aspects of HIV assembly (3, 16–19, 26, 34, 35, 40, 52). Maturation is accompanied by changes that include condensation of the NC-RNA complex to an electron-dense nucleoid and translocation of CA (p24) from a peripheral position to a more internal position relative to the viral membrane (15). A particle with the central nucleoid but in which CA protein remained in its peripheral location is not infectious (19). This suggests a role for the morphologically correct viral core in virus infectivity and implies that it would be possible to have an anti-HIV therapy based on intervention with the assembly of this core structure.

Rossmann (47) has suggested that the bacillus-shaped particles of alfalfa mosaic virus (12) may provide a structural model for the bacillus- or cone-shaped capsid of HIV. For alfalfa mosaic virus and several other unenveloped plant and animal viruses, in vitro studies with purified proteins have provided useful insight into the mechanism of virus coat assembly (12, 13, 20, 21, 45, 48). Similar studies with purified HIV CA proteins could provide information on the assembly of the capsid shell that would complement that obtained from genetic and ultrastructural studies. Such studies have been hampered by the lack of purified native CA protein, which was compounded by the lack of functional assays by which to assess the native status of the protein. Our abilities to express Gag-PR that is efficiently processed to mature

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products by HIV PR and to purify CA protein using a nondisruptive protocol (11) permit the undertaking of these in vitro studies. This recombinant CA protein has been crystallized as a CA-Fab complex as part of our effort to solve its three-dimensional structure (44). In this report, we present evidence that the recombinant CA protein alone can direct the formation of particles with a specific morphology. Moreover, we describe the results of experiments that probe the nature of CA-CA protein interactions in the oligomers that compose these particles.

MATERIALS AND METHODS

DNA constructs. Construction of the plasmid FS II was described previously (27). This plasmid contains a T7 promoter followed by HIV sequences from BH10 spanning nucleotides 221 to 2130 which includes 113 nucleotides of the 5′ nontranslated region, the *gag* gene, and that part of the *pol* gene that encodes proteinase. Four bases have been inserted at the *Bgl*II site in the early region of the *pol* gene just 5 bp downstream of the natural frameshift site. This results in a frameshift mutation and translation of the *pol* gene. In bacterial strains expressing the T7 RNA polymerase, FS II allows synthesis of Gag-PR, a truncated form of the Gag-Pol polyprotein.

Expression in *Escherichia coli*. Expression of the HIV sequence in FS II was done in *E. coli* BL21(DE3) as described before (11). Briefly, plasmid DNA was transformed into competent cells and transformed cells were grown in minimal media in a 14-liter fermentor (Microferm MMF-14). Expression of FS II was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. After 2 h of induction, cells were collected by centrifugation, and the wet paste was stored at −80°C until needed.

Purification of HIV type 1 (HIV-1) capsid protein. Recombinant CA protein expressed in BL21 (DE3) cells was purified essentially as described before (11). Briefly, 10 g of frozen cell paste was resuspended in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.5, with 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM EDTA and lysed by using a French press (SLM Instruments). The bacterial lysate was clarified by centrifugation at 10,000 × *g* for 15 min, and the supernatant was further centrifuged for 60 min at 200,000 × *g*. Proteins from the final supernatant were precipitated with 30% ammonium sulfate. The precipitate was collected by centrifugation, redissolved in 50 mM Tris-HCl buffer, pH 8, containing 30 mM NaCl and 1 mM EDTA, and chromatographed on a Whatman DE52 DEAE-cellulose column which had been equilibrated with the same buffer. Unbound proteins in the flowthrough fraction were precipitated by the addition of ammonium sulfate to 50% (wt/vol) and resuspended in 50 mM Tris-HCl buffer, pH 8, containing 30 mM NaCl and 1 mM EDTA to a protein concentration of about 30 mg/ml (typically, this required about a 1-ml volume). This solution was left unperturbed in a cold box overnight. The white precipitate of CA proteins that formed was collected and resuspended in a minimal volume of storage buffer (50 mM Tris-HCl buffer, pH 8, containing 30 mM NaCl). Four milligrams of purified CA protein was usually obtained from 10 g of bacterial cell pellet.

Protein analyses. Concentrations of protein samples were measured by using the Bio-Rad protein dye binding assay (Bio-Rad). Protein samples were electrophoresed under denaturing conditions on Laemmli gels (28). Nondenaturing (or native) gels were cast similarly except that these did not have the stacking portion, and sodium dodecyl sulfate (SDS) was omitted from both the separating gel and the running buffers. Electrophoresis on nondenaturing gels was done in a cold box. Gels were typically 12.5% acrylamide, unless otherwise indicated in the figure legends. Protein bands were visualized by staining with Coomassie blue. Immunological analysis of expressed proteins was done either by precipitation with specific antibodies followed by SDS-polyacrylamide gel electrophoresis (PAGE) or by immunoblotting using a procedure essentially as described by Towbin et al. (50). Automated Edman degradation of electroblotted proteins was performed by using a gas-phase Protein Sequencer model 470A equipped with a PTH Analyzer model 120A (Applied Biosystems) with standard programs recommended by the manufacturer.

Trypsin digestion. Digestion occurred in a 20-μl reaction mixture containing 3 μg of purified recombinant CA protein and variable amounts of trypsin (Boehringer Mannheim) in 100 mM Tris-HCl buffer, pH 8.5. Reaction mixtures were incubated at 37°C for either 30 or 60 min. Control reaction mixtures not containing trypsin were run in parallel. Both control and trypsin-treated samples were analyzed by electrophoresis on polyacrylamide gels under either denaturing or nondenaturing conditions.

Chemical cross-linking. Purified CA protein was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) prior to its use in a 14-μl reaction mixture containing buffer, 24 μg of purified CA protein, and cross-linking agent. Reactions were carried out at room temperature and stopped by the addition of glycine to a final concentration of 100 mM. Reaction products were separated by electrophoresis on SDS-polyacrylamide gels and detected by Western immunoblot analysis with anti-p24 antibody. The cross-linking agents used included sulfoethylene glycolylbis(succinimidylsuccinate), dithiobis(succinimidylpropionate), and 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) (1, 32, 49).

Gel filtration column chromatography. Chromatography of purified CA protein was done on a Sephadex G-200 column (1.0 by 40 cm) which was equilibrated and eluted with buffer (50 mM Tris-HCl, pH 8.0, containing 30 mM NaCl and 1 mM EDTA) at a flow rate of 6 ml/h. The column was calibrated with a protein mixture (200 μl) consisting of Blue Dextran (2×10^6 kDa), bovine serum albumin (BSA) (68 kDa), chymotrypsinogen (25 kDa), and cytochrome *c* (13 kDa). After the column was washed with 500 ml of buffer, a 200-μl solution of CA protein at 10 mg/ml was loaded and chromatographed in identical fashion. After another round of washing, a second 200-μl sample containing CA protein at 1 mg/ml was loaded and chromatographed as described before. For all three chromatographic runs, eluate was collected at 1 ml per fraction. Fractions containing CA or standard proteins were identified following SDS-PAGE of 20-μl aliquots of the fractions collected.

Electron microscopy. Samples were negatively stained with 2% uranyl acetate and visualized by using a JEOL 1200 EX electron microscope.

RESULTS

Properties of recombinant HIV-1 capsid protein. The HIV Gag-PR polyprotein that is encoded by plasmid FS II (11, 27; also Materials and Methods) was synthesized and efficiently processed in BL21(DE3) cells. Expressed CA protein was purified by using a protocol developed in our laboratory that includes in vitro oligomerization in the final step. The protocol is described briefly in Materials and Methods and in

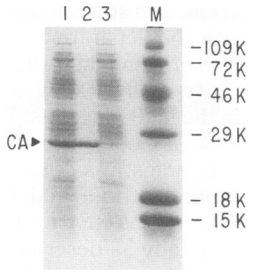

FIG. 1. Isolation of recombinant HIV-1 CA proteins from *E. coli*. Recombinant CA was isolated by a French press of induced cells carrying FS II. The lysate was clarified by centrifugation and chromatographed on a DEAE-cellulose column as described in Materials and Methods. Proteins in the unbound column fraction were concentrated to ~30 mg/ml by precipitation with ammonium sulfate and kept at 5°C overnight. Under these conditions, recombinant CA proteins separate as a white precipitate that readily redissolves in low-ionic-strength buffer. The figure shows a Coomassie-stained SDS-polyacrylamide gel containing proteins in the unbound fraction of the DEAE-cellulose column (lane 1), precipitated recombinant CA redissolved in buffer (lane 2), and proteins remaining in the supernatant after centrifugation of precipitated CA (lane 3). Molecular weights are on the right.

greater detail in an earlier report (11). The isolation of CA protein in the final stage of the purification is illustrated by the Coomassie-stained gel shown in Fig. 1. Lane 1 in Fig. 1 shows the unbound proteins that coeluted with CA in the flowthrough fraction after chromatography on DEAE-cellulose. These proteins were concentrated by ammonium sulfate precipitation, resuspended at about 30 mg/ml (total protein concentration), and left unperturbed at 5°C. CA protein separated as a white precipitate (Fig. 1, lane 2), leaving most of the other proteins in the supernatant (Fig. 1, lane 3).

The CA protein expressed in bacteria shares several properties with CA protein isolated from the virus. The recombinant CA preparation consists of two isoelectric forms that exhibit pI values of 6.55 and 6.75. The pI = 6.75 form predominated in most preparations (44; also data not shown). Both of these pI values are similar to those reported for capsid protein found in mature virus (pI = 6.6 and pI = 6.7; for a review, see reference 30). Recombinant CA protein was recognized in the native form by monoclonal and polyclonal antibodies directed against viral CA protein in immunoprecipitation experiments (data not shown). Analysis by Edman degradation and digestion with carboxypeptidase P (data not shown) indicated that both the N- and C-terminal amino acid sequences of the recombinant CA protein were identical to that of CA protein isolated from virus (22).

Concentration-dependent dimer formation. Observations that AKR murine leukemia virus (41, 43), Moloney murine leukemia virus (4), avian myeloblastosis virus (42), or recombinant HIV CA (11, 37) proteins exhibit self-associative properties have been previously reported. To identify stable multimers that might give insight into the mechanism of capsid shell assembly, we measured the molecular size of CA protein under various conditions in vitro. Samples of purified recombinant CA protein at 1 or 10 mg/ml were chromatographed on a Sephadex G-200 column that had been equilibrated with 50 mM Tris-HCl buffer, pH 8, containing 30 mM NaCl and 1 mM EDTA. The elution positions of the CA polypeptides and the protein markers used to calibrate the column were determined by SDS-PAGE analysis of aliquots of collected fractions. Panel A in Fig. 2 is a Coomassie-stained gel of fractions collected during column calibration and shows the elution positions of BSA (M_r = 68,000), chymotrypsinogen (M_r = 25,000), and cytochrome *c* (M_r = 13,000) standards. The position of Blue Dextran was located spectrophotometrically and is indicated. Figure 2B shows the elution position of the 10 mg/ml CA protein sample. Under these conditions, CA protein eluted slightly behind BSA (M_r = 68,000) and ahead of chymotrypsinogen (M_r = 25,000). Assuming that the protein is globular, this pattern is consistent with the molecular size of a CA protein dimer (50 kDa). This conclusion was supported by the results obtained upon dilution of the protein. Figure 2C shows the elution profile of CA when chromatographed at 1 mg/ml. The CA protein eluted as a monomer with an estimated molecular mass of about 25 kDa. The elution of CA protein as a monomer when chromatographed as a 1 mg/ml solution and as a dimer when chromatographed as a 10 mg/ml solution reflects the dependence of CA dimer formation on protein concentration. Similar results were obtained when the recombinant CA protein was sedimented through a 4 to 40% sucrose density gradient (data not shown).

CA protein dimers cross-link in nonrandom arrays. Chemical cross-linking agents were used to determine whether the recombinant CA protein dimers were organized in larger structures. Cross-linkers capable of bridging distances between arrays should permit determination of higher-order structure. The results of cross-linking studies using DTSSP (span length = 1.2 nm) are shown in Fig. 3. CA protein (1.7 mg/ml) was incubated with increasing concentrations of cross-linking agent for 60 min prior to analysis of reaction products by Western analysis. In the absence of DTSSP, most of the CA protein migrated with an M_r of 25,000 (Fig. 3, lane 5). A faint band (M_r = 50,000) was also visible. This subpopulation of dimeric CA protein was not observed consistently and may have been due to occasional oxidization of sulfhydryl groups leading to the formation of intermolecular disulfide bridges. Increasing amounts of cross-linked CA molecules that migrated with M_rs of ~50,000 were detected with increasing amounts of DTSSP (Fig. 3, lanes 1 to 4). It is likely that some or all of the proteins detected as monomers under these conditions were involved in nonproductive interactions because of hydrolysis of one of the two reactive groups in the reagent prior to binding to a second CA molecule. At the highest concentration of DTSSP (Fig. 3, lane 4), dimers, tetramers, and monomers were detected in excess over trimers and higher oligomers. Similar results were obtained in studies using two other bifunctional cross-linking agents, sulfoethylene glycolylbis(succinimidylsuccinate) and dithiobis(succinimidylpropionate) (data not shown). Since random collision would generate more trimers than tetramers (20), the results suggest that CA protein dimers form the basic subunit of higher-order structures. Cross-linked dimers, tetramers, and higher-molecular-weight forms also were observed in previous studies using native particles of avian myeloblastosis virus (42) and murine leukemia virus (43).

Recombinant HIV capsid protein can form higher-ordered oligomers. The fact that tetramers and higher oligomers of CA protein were detected by chemical cross-linking but not be gel filtration or sedimentation analyses suggested that CA protein association states might be unstable or reversible under some experimental conditions. The migration of recombinant CA in nondenaturing polyacrylamide gels sup-

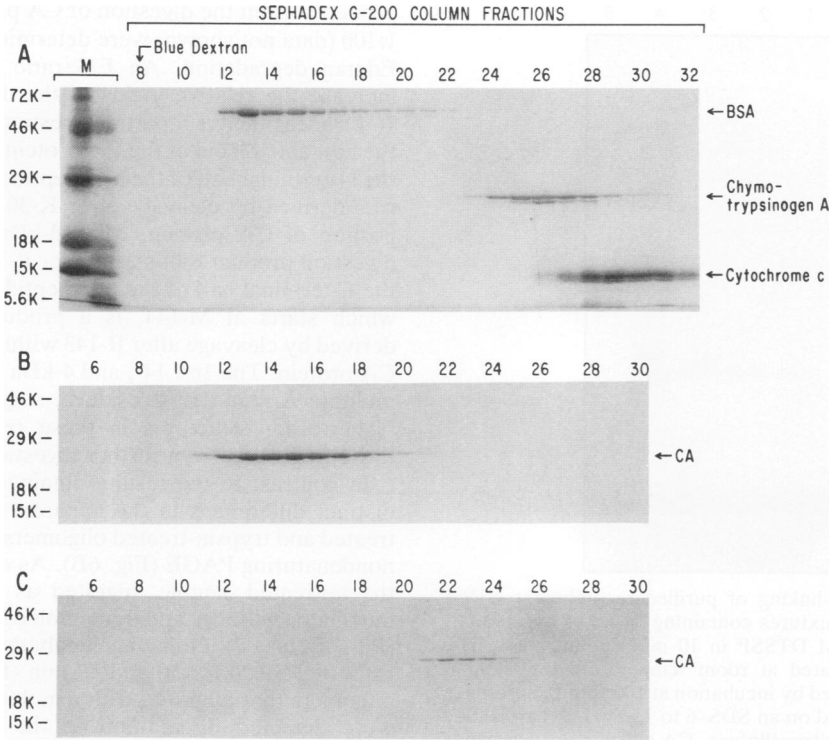

FIG. 2. Gel filtration of purified recombinant HIV-1 capsid protein. Two-hundred-microliter samples of a 10 mg/ml and a 1 mg/ml solution of purified recombinant CA protein were chromatographed on a Sephadex G-200 column that was preequilibrated with 50 mM Tris (pH 8)–30 mM NaCl buffer. Column eluates were collected, and 20-μl aliquots were analyzed for CA protein by electrophoresis on SDS–12.5% polyacrylamide gels. (A) Gel showing the elution positions of the following column calibration protein standards: monomeric BSA (molecular weight [MW] = 68,000), chymotrypsinogen (MW = 25,000), and cytochrome *c* (MW = 13,000). (B) Gel showing the elution position of CA applied to the column as a 10 mg/ml solution. (C) Gel showing the elution position of CA protein applied as a 1 mg/ml solution. Molecular weights for each panel are on the left.

ported this view (Fig. 4). Denatured CA protein migrated with the expected molecular mass of 24 kDa, as shown in Fig. 4A. When analyzed under nondenaturing conditions (Fig. 4B and C), the CA protein migrated as large oligomers. At a protein concentration of 2 mg/ml, the CA protein migrated as a broad band with an apparent molecular mass of approximately 300 kDa (Fig. 4B). At a protein concentration of 5 mg/ml, CA protein exhibited an apparent molecular mass that was greater than that of the 545-kDa urease marker (Fig. 4C). Oligomers that comigrated with the ferritin marker (~800 kDa) have been observed with CA protein preparations at concentrations of 10 mg/ml or higher (data not shown). Smaller amounts of oligomers that were approximately the size of trimers or dimers also have been detected (Fig. 4; also see Fig. 5 below). We interpret the presence of a species with an M_r of about 300,000 to suggest that the CA protein dimers formed dodecamers which were stable under these conditions (predicted M_r = 288,000). The ~300-kDa oligomer was stable to dilution and was still detected at protein concentrations as low as 100 μg/ml (data not shown). The detection of multimers of this ~300-kDa oligomer with increasing protein concentration suggests that these form repeating units (predicted M_r = 576,000, 864,000, etc. [38]).

CA protein oligomers are stable to high-ionic-strength and nonionic detergents. We examined the effects of high-salt and nonionic detergents on the stability of CA protein interactions. Figure 5A shows the migration of CA proteins in 6 to 15% nondenaturing gels after incubation in buffers containing 1 M NaCl or a nonionic detergent (0.1% octyl-beta glucopyranoside) (OBG). The CA protein in the untreated sample migrated as two major oligomeric species with apparent molecular masses of >545 and ~300 kDa and as two minor species of ~200 and ~70 kDa (Fig. 5B). The two major species were detected in a ratio of 2:1 (>545:300 kDa). Incubation of the protein with NaCl or OBG altered this molecular size distribution. The ratio of the >545- and 300-kDa forms decreased to 1:1, and smaller oligomers migrating at about 250 and 132 kDa appeared. Neither monomers nor dimers were detected. The new molecular size distribution indicated that both salt and detergent promoted small degrees of dissociation. However, this limited dissociation occurred only after several weeks of incubation, indicating that the oligomers were actually quite stable under these conditions.

Oligomerization is affected by limited proteolysis. Since the cross-linking experiments described above indicated the presence of residues with accessible primary amino groups in or near regions of protein-protein interaction, we examined the possibility that limited proteolysis with trypsin affects CA protein oligomerization. Trypsin cleaves on the carboxy side of amino acids with primary amino groups. In contrast to the minimal effects of high salt concentration and nonionic detergent, the incubation of CA proteins with trypsin even at a low enzyme-to-substrate (E/S) ratio resulted in dramatic changes in the apparent size of the oligomers (Fig. 6). Panel A in Fig. 6 shows SDS-PAGE of untreated CA (Fig. 6A, lane 7) and CA protein which was incubated with increasing amounts of trypsin (Fig. 6A, lanes

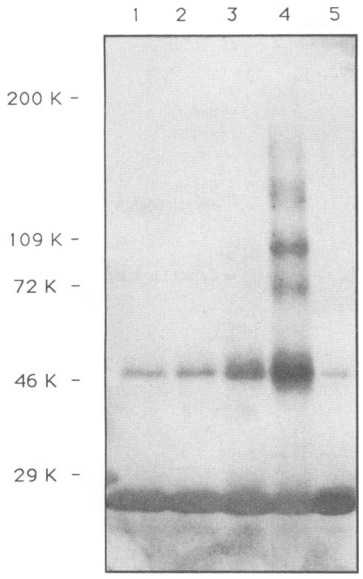

FIG. 3. Chemical cross-linking of purified recombinant HIV-1 capsid protein. Reaction mixtures containing 24 μg of purified CA protein and 0.01 to 10 mM DTSSP in 10 mM sodium phosphate buffer (pH 7) were incubated at room temperature for 60 min. Proteins were then denatured by incubation at 100°C in the presence of 1% SDS, electrophoresed on an SDS–6 to 12.5% polyacrylamide gel, and transferred onto nitrocellulose. CA-specific protein bands were detected by probing with anti-CA monoclonal antibodies. The figure shows an immunoblot of reaction mixtures incubated with 0.01 (lane 1), 0.1 (lane 2), 1.0 (lane 3), and 10 (lane 4) mM DTSSP. CA protein incubated for 60 min without DTSSP is shown in lane 5.

1 to 6). Protein was visualized with Coomassie blue stain. Limited proteolysis occurred under all conditions tested, resulting in a minor population of fragments that migrated at 2.5, 4, 14, and 16 kDa. The majority of the CA protein in all samples comigrated with the CA protein in the untreated sample.

To identify the sites cleaved by trypsin, the N-terminal amino acids of the 2.5-, 4-, 14-, and 16-kDa fragments produced from the digestion of CA protein at an E/S ratio of 1:100 (data not shown) were determined by using automated Edman degradation. An E/S ratio of 1:100 was used to increase the relative yields of the digestion products. The 16-kDa fragment is a partial digestion fragment that starts at the first amino acid of the CA protein (P) and thus represents the N-terminal half of the polypeptide. The 2.5-kDa fragment was derived by cleavage after K-30 within this N-terminal portion of CA protein. The 14-kDa fragment is a partial digestion product that starts after R-100 and thus represents the C-terminal half of the polypeptide. The 4-kDa fragment, which starts at M-144, is a product of partial digestion derived by cleavage after R-143 within this C-terminal half of CA protein. The 16-, 14-, and 4-kDa fragments all contained multiple Arg and Lys residues, suggesting the presence of higher-order structures in these regions which protected these fragments from further digestion.

In contrast to the results obtained with denaturing gels, distinct differences in the apparent molecular sizes of untreated and trypsin-treated oligomers were detected by using nondenaturing PAGE (Fig. 6B). As expected, CA protein in the untreated sample migrated as a single population of oligomers with an apparent molecular mass of ~300 kDa (Fig. 6B, lane 7). Following incubation with trypsin at an E/S ratio of 1:5,000 for 30 and 60 min (Fig. 6B, lanes 1 and 2), oligomers that migrated with a molecular mass of ~200 kDa were detected in addition to the ~300-kDa species. In samples treated at higher E/S ratios (i.e., 1:500 and 1:1,000), only the smaller oligomeric form was detected. To determine whether this smaller oligomeric form represented an association of truncated CA molecules, this band was electroblotted onto a membrane of polyvinylidene difluoride (Immobilon) for analysis of the N terminus by automated Edman degradation or was eluted for analysis of the C terminus by hydrolysis with carboxypeptidase P followed by amino acid analysis. Evidence for neither amino- nor carboxy-terminal hydrolysis was obtained by these analyses (data not shown). These data are consistent with the interpretation that limited trypsin hydrolysis resulted in the removal of four CA protein molecules (~100 kDa) from the 300-kDa oligomer. It is possible that the interactions between subunits within the

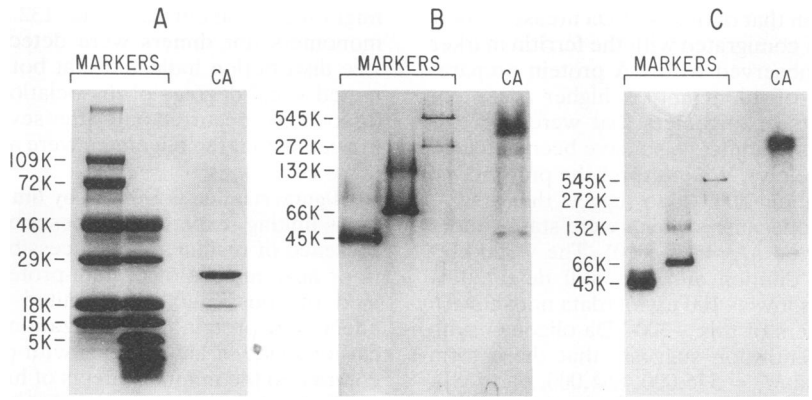

FIG. 4. Migration of purified recombinant HIV-1 on native polyacrylamide gels. Twenty-microgram samples of purified recombinant CA were electrophoresed on Laemmli gels under denaturing and nondenaturing conditions. Proteins were visualized by staining with Coomassie blue. (A) A denaturing 10 to 20% polyacrylamide gradient gel with 20 μg of CA protein from a 2 mg/ml solution. (B) A nondenaturing 10 to 20% polyacrylamide gel with 20 μg of CA protein from a 2 mg/ml solution. (C) A nondenaturing 4 to 20% polyacrylamide gel with 20 μg of CA protein from a 5 mg/ml solution. Molecular size markers: ovalbumin (MW = 45,000), BSA monomer (MW = 66,000), BSA dimer (MW = 132,000), urease trimer (MW = 272,000), and urease hexamer (MW = 545,000).

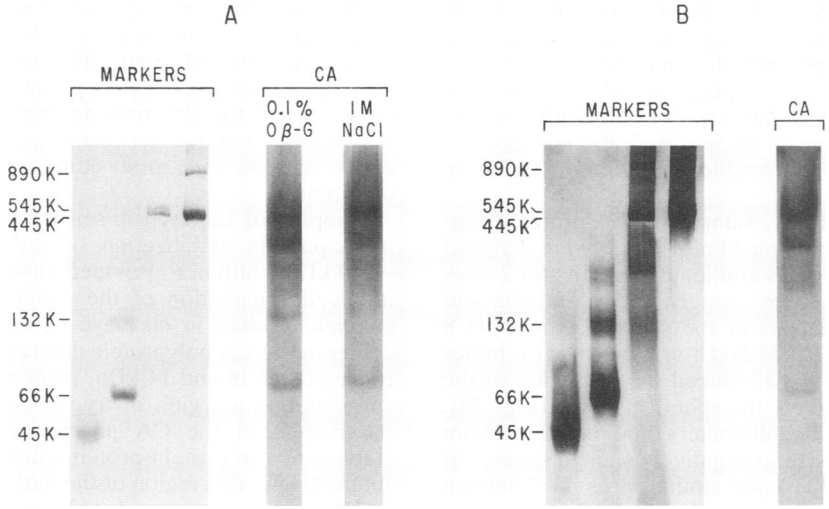

FIG. 5. Migration of purified recombinant CA protein on nondenaturing gels after treatment with 1 M NaCl and 0.1% OBG. NaCl and OBG were added to aliquots of a 2 mg/ml solution of purified recombinant CA. These samples were kept at 5°C for several weeks prior to electrophoresis on a 6 to 15% acrylamide gel under nondenaturing conditions. Protein bands were visualized by staining with silver. (A) Markers and samples containing 20 μg of CA protein with 1 M NaCl or 0.1% OBG; (B) markers and 20 μg of untreated CA protein. Molecular weight markers: ovalbumin (MW = 45,000), BSA monomer (MW = 66,000), BSA dimer (MW = 132,000), jack bean urease hexamer (MW = 545,000), ferritin monomer (MW = 445,000), and ferritin dimer (MW = 890,000).

300-kDa oligomer are not identical and that susceptible regions exposed in one subunit were shielded in another.

Most likely, the observed change in molecular mass was attributable to digestion of accessible CA molecules into the 2.5-, 4-, 14-, and 16-kDa products detected in Fig. 6A. These minor products were identified on the native gel (Fig. 6C) by N-terminal and immunological analyses (data not shown). These fragments were not detected at the migration positions of the 300- or 200-kDa oligomers, as shown by N- and C-terminal analyses of the proteins in these oligomers and by excising the bands and subjecting them to reelectrophoresis under denaturing conditions in SDS gels (data not shown). The fact that neither the 16- nor 14-kDa fragment that spans the N-terminal and C-terminal halves of the CA polypeptide, respectively, was associated with the 200- or 300-kDa oligomers suggests that these putative structures were insufficient for, or not involved in, maintaining oligomer association. Perhaps the concerted action of structural domains located in both halves of the CA polypeptide is required. Alternatively, cleavage may have effected conformational changes in regions of the protein involved in oligomer association. In either case, these results indicate that the structural integrity of a region in the middle of CA protein is required for oligomer association.

Morphology of structures formed by recombinant HIV capsid protein in vitro. Purified coat proteins of tobacco mosaic virus, polyomavirus, and several simple spherical plant viruses have been observed to form functionally relevant structures in vitro (20, 45, 48). To determine whether the recombinant CA protein oligomers participate in the formation of higher-ordered structures in vitro, samples of the purified protein were examined by using negative stain-

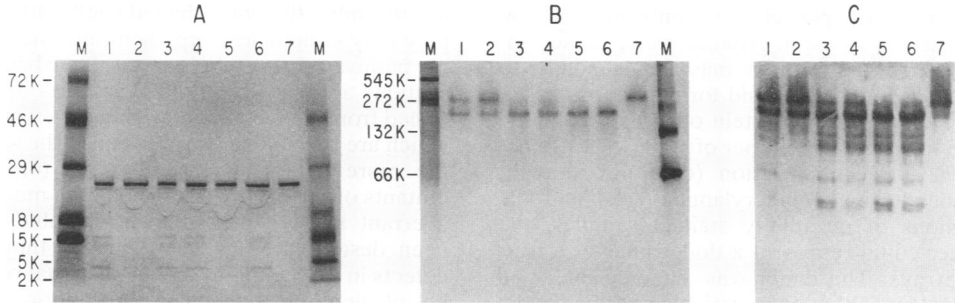

FIG. 6. Migration of CA protein on native gels after limited trypsin digestion. Tryptic digestion was carried out in reaction mixtures containing 100 mM Tris-HCl buffer (pH 8.5), 3 μg of purified recombinant CA protein, and 0.6, 3.0, or 6.0 ng of trypsin. Each mixture was made in triplicate and incubated at 37°C for 30 or 60 min. (A) Electrophoresis of trypsin-treated samples in 10 to 20% denaturing polyacrylamide gradient gels. Protein was visualized by Coomassie blue staining. (B) Electrophoresis in 10 to 20% nondenaturing polyacrylamide gradient gel. Proteins were visualized as described for panel A. (C) An immunoblot of the trypsin-treated samples analyzed on a nondenaturing polyacrylamide gel probed with polyclonal anti-CA antibody. Lanes: 1, incubation with 0.6 ng of trypsin (E/S = 1:5,000), 60 min; 2, incubation as in lane 1, 30 min; 3, incubation with 3 ng of trypsin (E/S = 1:1,000), 60 min; 4, incubation as in lane 3, 30 min; 5, incubation with 6 ng of trypsin (E/S = 1:500), 60 min; 6, incubation as in lane 5, 30 min; 7, CA protein incubated for 60 min in the absence of trypsin; M, molecular weight markers.

ing and electron microscopy. The most abundant structure detected resembled flexible elongated fibers of variable length (~200 nm and longer) with frequent points of branching and bending (Fig. 7A). The preparation also contained minor amounts of other structures which were either spherical or rodlike in appearance. These observations suggested that the CA protein was capable of different assembly pathways in vitro.

To examine this possibility, samples of CA protein (originally in pH 8 buffer containing 30 mM NaCl) were dialyzed against buffers of various pHs and ionic strengths for 2 days at 5°C and then examined by using negative staining and electron microscopy. Dialysis of the sample against pH 8 buffer containing 0.1 to 1.0 M NaCl or against pH 6 buffer containing 0.1 to 0.3 M NaCl induced the formation of the structures having exclusively the spherical shape (Fig. 7B). These imperfect spheres had diameters of about 10 to 20 nm and were comparable in size to negatively stained spheres of horse ferritin (440 kDa [33]) and simian virus 40 T-antigen hexamers (556 kDa [34]). By analogy to horse ferritin, these structures may represent the oligomers detected under nondenaturing conditions (Fig. 4). This suggests that the flexible fibers present in the original CA protein preparation were dissociated under these conditions and that the smaller spheres are the unassembled form of these structures. Dialysis of the original sample against pH 6 buffer that contained 1.0 M NaCl induced the formation of structures with predominantly rigid rod shapes (Fig. 7C). These were distinctly more organized and structured than the flexible fibers and spheres. Although the rodlike structures also had the tubular shape of the flexible fibers, they lacked the bends and branches that characterized the fibers and were of uniform width (~10 nm). The addition of divalent cations such as Zn^{2+}, Mg^{2+}, and Ca^{2+} to dialysis buffers did not change the morphology of the structures detected (data not shown). The results suggest that the conformation of the CA protein and its capacity for interactions at its surface can be altered by specific in vitro conditions.

DISCUSSION

The results described in this report indicate that recombinant HIV CA protein can form stable oligomeric structures under different conditions in vitro. These observations indicate that associative properties are intrinsic to the CA protein. It may be that the highly multimerized state in which Gag is found in immature particles is contributed to by interactions through this domain. Alternatively, these biochemical properties of CA protein may relate solely to interactions involved in mature capsid formation.

The most stable forms of CA protein oligomers detected were a dimer (~48 kDa) and a multimer of ~300 kDa. On the basis of analytical ultracentrifugation (data not shown), migration in nondenaturing polyacrylamide gels, and the molecular dimensions of negatively stained samples, the ~300-kDa multimer could represent a dodecamer or a hexamer of dimeric arrays. The dimer was detected under all experimental conditions, although in variable relative yields. In contrast, the ~300-kDa multimer was detected only under conditions of minimal sample perturbation. The liability of this multimer under conditions of sucrose density gradient sedimentation or gel filtration chromatography on Sephadex columns contrasts with its stability to high-ionic-strength or nonionic detergent and suggests that the structure may be destabilized by interactions with sucrose or components in the gel matrix. The influence of ligands on the equilibrium of the various oligomeric species of a number of self-associating proteins is well documented (39). It is not known whether the association and dissociation of CA oligomers are similarly ligand influenced. It is possible that the various techniques used for determining molecular size distribution introduce or remove pertinent ligands from the CA sample under analysis and, in so doing, affect the molecular size distribution.

Mapping of the trypsin-sensitive regions that appear to participate in stabilization of the dimeric arrays in the ~300-kDa multimer provided insight into the sequences involved. Incubation of the ~300-kDa oligomer with the enzyme resulted in cleavage of an exposed region in the center of the CA polyprotein and released N- and C-terminal fragments of 16 and 14 kDa, respectively. This observation suggests that a region of trypsin susceptibility localized in the middle of the CA polypeptide plays a role in the stabilization of protein-protein interactions in the multimer. Interestingly, this region of the molecule contains a segment which is highly conserved in all retroviruses (51). This major homology region could play a role in protein-protein interactions of the CA protein. One of the trypsin-susceptible sites that we have mapped is located in a region predicted to be exposed in a model of CA protein proposed by Argos (2). Paradoxically, none of several anti-CA monoclonal antibodies recognized this region (29). Perhaps its involvement in protein-protein interactions accounts for this observation.

The fiberlike structures identified by using electron microscopy presumably resulted from the assembly of the ~300-kDa multimer subunits. Flexible fibers and rigid rods were readily formed and dissociated by adjustment of the protein concentration, the pH, or the ionic strength of the solution. The sensitivity of these higher-order structures to such factors indicates that polar interactions on the multimer surface regulate their formation. The results also suggest that capsid shell assembly occurs through association of preassembled intermediates rather than by direct polymerization of monomeric or dimeric forms of the capsid protein. An assembly pathway that utilizes preassembled subunits would permit strict regulation of morphogenesis by environmental factors or other *gag* gene products.

In mature HIV particles, the CA protein forms a cone-shaped capsid shell 120 to 130 nm in length, 55 to 62 nm in diameter on the broad end, and 25 nm in diameter on the tapered end (14). In contrast, the structures we assembled in vitro exhibited tubular shapes. Although these structures do not resemble the cone-shaped shell found in virus particles, they may nonetheless represent intermediates in the assembly pathway. Long tubular cores have been described in thin sections and negative-stained samples of viral particles obtained from HIV-infected cells (6, 14). These aberrant cores, which are variable in length, display the same antigenicity as the more abundant conical cores (6, 14). Interestingly, mutants of the bacillus-shaped alfalfa mosaic virus that form aberrant tubular structures of variable lengths also have been described. This phenotype is thought to be due to defects in the switching mechanism that regulates the assembly of dimeric subunits into hexameric arrays in the cylindrical body and into pentameric arrays in the icosahedral ends (7, 12). RNA directly regulates this switching mechanism by altering the conformation of the N-terminal segment of the coat protein. In the case of HIV, formation of the cone-shaped shell also may require alternate conformations of CA protein induced by interaction with nucleic acid, other Gag proteins, or posttranslational modifications. Experiments with purified CA protein in vitro provide an approach

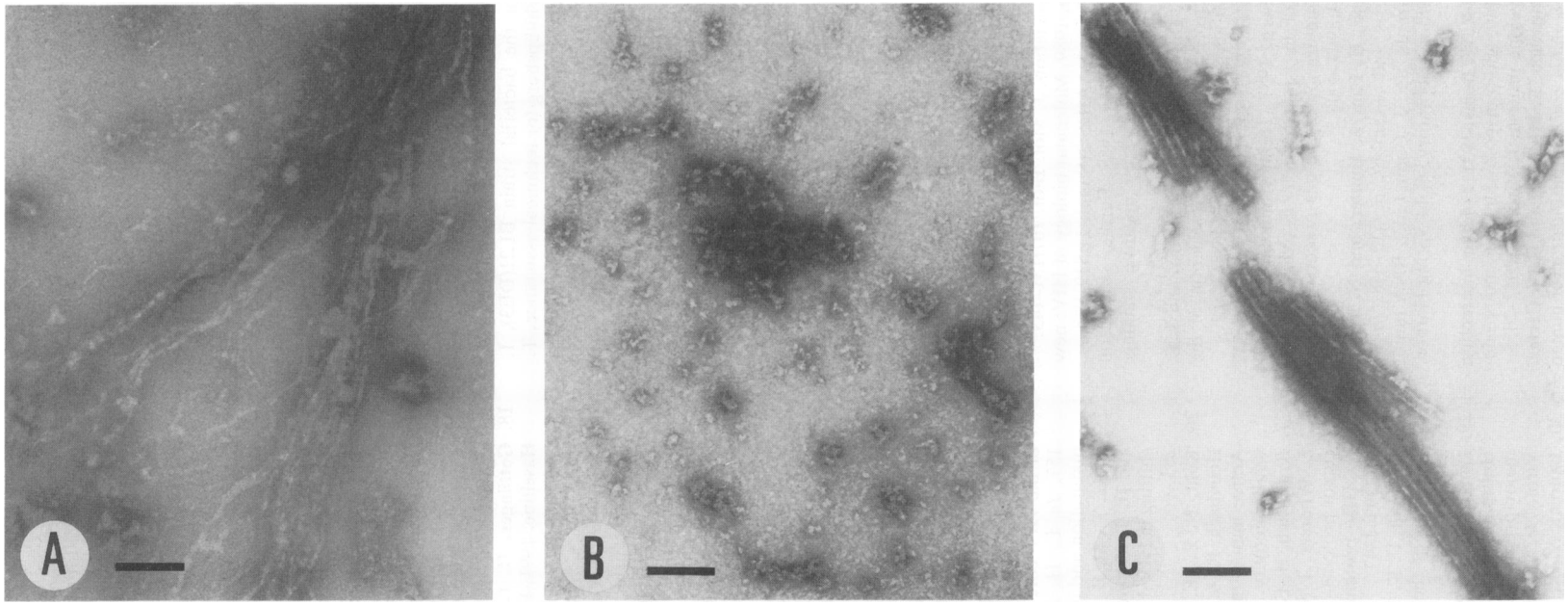

FIG. 7. Electron microscopy of negatively stained samples of purified recombinant HIV-1 capsid protein. One-hundred-microliter aliquots of purified CA protein (10 mg/ml) were dialyzed against 300 ml of 50 mM Tris (pH 8) or acetate (pH 6) buffer of different ionic strengths for 2 days at 5°C. Dialyzed samples were applied to copper grids and stained with 2% uranyl acetate. (A) Electron micrograph of undialyzed CA preparation protein in 50 mM Tris-HCl buffer (pH 8)–30 mM NaCl. (B) A representative electron micrograph of CA protein after dialysis under any of the following conditions: pH 8, 0.1 M NaCl; pH 8, 0.3 M NaCl; pH 8, 1.0 M NaCl; pH 6, 0.1 M NaCl; pH 6, 0.3 M NaCl; pH 6, 0.1 M NaCl with 1 mM $CaCl_2$, $MgCl_2$, or $ZnCl_2$; pH 8, 0.1 M NaCl with 1 mM $CaCl_2$, $MgCl_2$, or $ZnCl_2$. (C) Electron micrograph of CA protein after dialysis against pH 6 buffer containing 1.0 M NaCl. Magnification, ×40,000. Bars, 200 nm.

for the manipulation of assembly conditions to test the role of such factors in capsid shell assembly.

In summary, we have demonstrated that purified recombinant HIV-1 CA protein has self-associative properties which provide a basis for a capsid shell assembly process driven by CA-CA protein association. We have shown that CA proteins in vitro can form dimers that associate into oligomers. Our evidence suggests that a central region of the protein which is accessible to trypsin is involved in stabilization of the protein-protein interactions required for oligomer formation in vitro. The association of oligomers into larger structures and the dissociation of the larger structures can be induced readily upon dilution or changes in ionic strength and pH. The results suggest that the HIV capsid assembly process involves the association of preassembled subunits. The aberrant morphology of structures obtained in vitro suggests the participation of control mechanisms in the assembly of the characteristic cone-shaped lentiviral core.

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